

# Unprecedented Mechanism of Chain Length Determination in Fungal Aromatic Polyketide Synthases

Akira Watanabe and Yutaka Ebizuka\*

School of Pharmaceutical Sciences  
The University of Tokyo  
Bunkyo-ku, Tokyo 113-0033  
Japan

## Summary

Fungal aromatic polyketides show remarkable structural diversity fundamentally derived from variations in chain length and cyclization pattern. Their basic skeletons are synthesized by multifunctional iterative type I polyketide synthases (PKSs). Recently, we have found that the C-terminal thioesterase (TE)-like domain of *Aspergillus nidulans* WA catalyzes Claisen-type cyclization to form the B-ring of naphthopyrone YWA1. Here we report the unprecedented mechanism of chain length determination by the C-terminal TE-like domain of *Colletotrichum lagenarium* PKS1, which, in addition to catalyzing Claisen-type cyclization, intercepts the polyketomethylene intermediate from the acyl carrier protein domain during the condensation reaction to produce shorter chain length products. This chain length determination system is novel among PKSs, including bacterial and plant PKSs. The functional diversity of the TE-like domain directly influences the structural diversity of aromatic polyketides in *C. lagenarium* PKS1.

## Introduction

The polyketides are an important group of natural products and a source of biologically active compounds. Polyketides are synthesized from several acyl units by polyketide synthases (PKSs), which catalyze successive condensation reactions of acyl-CoAs, mostly acetyl- and malonyl-CoAs, to produce polyketomethylene intermediates and some subsequent reactions such as cyclization and reduction of the intermediates. PKSs are separated into three types: type I [1, 2], type II [3], and type III [4] PKSs. Type I PKSs are further divided into two classes: modular type I PKSs and iterative type I PKSs.

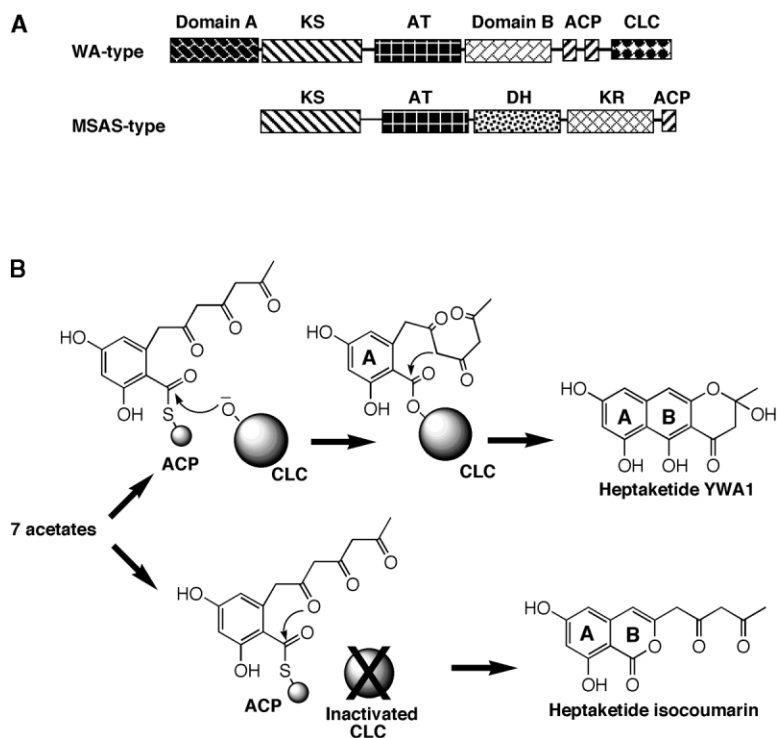
Many investigations have been carried out to demonstrate how PKSs control reactions such as chain length determination in plant and bacterial PKSs. Bacterial modular type I PKSs, which produce macrolides such as erythromycin, are composed of several modules that each catalyze a single condensation step. In modular PKSs, the product chain length is determined depending on the number of modules, and the product is lactonized by thioesterase (TE) domains. Bacterial type II PKSs, which produce polycyclic aromatic polyketides such as tetracycline, are composed of an assembly of discrete enzymes. A chain length factor (CLF), which is homologous to a  $\beta$ -ketoacylsynthase (KS), forms a heterodimer

with a KS. This KS-CLF complex, together with an acyl carrier protein (ACP) and a malonyl-CoA:ACP transacylase, makes up a so-called minimal PKS. Chain length of the type II PKS products is determined by this minimal PKS. The first ring of the product is formed in the minimal PKS reaction and discrete cyclases catalyze further cyclizations. Recently, the KS-CLF complex has been shown to possess decarboxylation activity toward malonyl-ACP as well to provide acetyl-ACP required to prime chain extension [5]. Plant and bacterial type III PKSs, which produce aromatic polyketides such as precursors of flavonoids and vancomycin, are homologous to *Escherichia coli* KS III. The crystal structure of plant chalcone synthase (CHS) has been elucidated [6], and the engineering of CHS based on that structural knowledge has demonstrated that the space of the active site cavity restricts product size [7]. However, compared with plant and bacterial PKSs, the mechanism of reactions catalyzed by fungal iterative type I PKSs is still unclear.

Fungal iterative type I aromatic PKSs are divided into two groups based on domain architecture (Figure 1A): the MSAS-type such as *Penicillium patulum* MSAS [8] and *Aspergillus terreus* ATX [9], and the WA-type such as *Aspergillus nidulans* WA [10] and *Colletotrichum lagenarium* PKS1 [11]. WA-type PKSs characteristically have an N-terminal extended domain (domain A), a central domain (domain B), and a C-terminal TE-like domain. Domain A and domain B have no homology to any other known functional proteins and their functions are still unknown. On the other hand, we have recently demonstrated that the C-terminal TE-like domain of *A. nidulans* WA functions as a Claisen cyclase (CLC) that catalyzes Claisen cyclization of the B-ring of heptaketide YWA1 [12, 13] (Figure 1B). When the WA CLC domain is inactivated, the B-ring is formed by nonenzymatic lactonization to produce heptaketide isocoumarin [14]. As other WA-type PKSs such as *C. lagenarium* PKS1 also produce Claisen cyclization-type compounds, the C-terminal TE-like domains of other WA-type PKSs are also expected to function as Claisen cyclases.

*C. lagenarium* PKS1 also has domain architecture identical to that of WA, although PKS1 produces multiple products, i.e., tetraketide orsellinic acid, pentaketide  $\alpha$ -acetylorsellinic acid, pentaketide tetrahydroxynaphthalene (THN) [15], and an unknown compound when heterologously expressed in *Aspergillus oryzae*, unlike WA that specifically produces the heptaketide YWA1 under the same heterologous expression condition. The production of  $\alpha$ -acetylorsellinic acid suggests that the C-terminal TE-like domain of PKS1 catalyzes the hydrolysis of an ACP-bound pentaketide intermediate as well as Claisen-type cyclization, unlike the WA CLC domain. Furthermore, we have recently reported that a chimeric PKS, SW-B, produces hexaketides as major products [16], while wild-type PKS1 produces pentaketides as major products. As SW-B is a PKS1 derivative in which the ACP and C-terminal TE-like domains are replaced with those of WA and there is no evidence that ACP controls the product chain length, the C-terminal TE-

\*Correspondence: yebiz@mol.f.u-tokyo.ac.jp



**Figure 1. C-Terminal Domains of Fungal PKSs**  
(A) Domain architecture of WA-type PKSs and MSAS-type PKSs. KS,  $\beta$ -ketoacylsynthase; AT, acyltransferase; ACP, acyl carrier protein; CLC, Claisen-type cyclase; KR,  $\beta$ -ketoacyl reductase; DH, dehydratase.  
(B) Claisen cyclization of the B-ring of YWA1 by the WA CLC domain.

like domain seems to be involved in the control of the product chain length in PKS1.

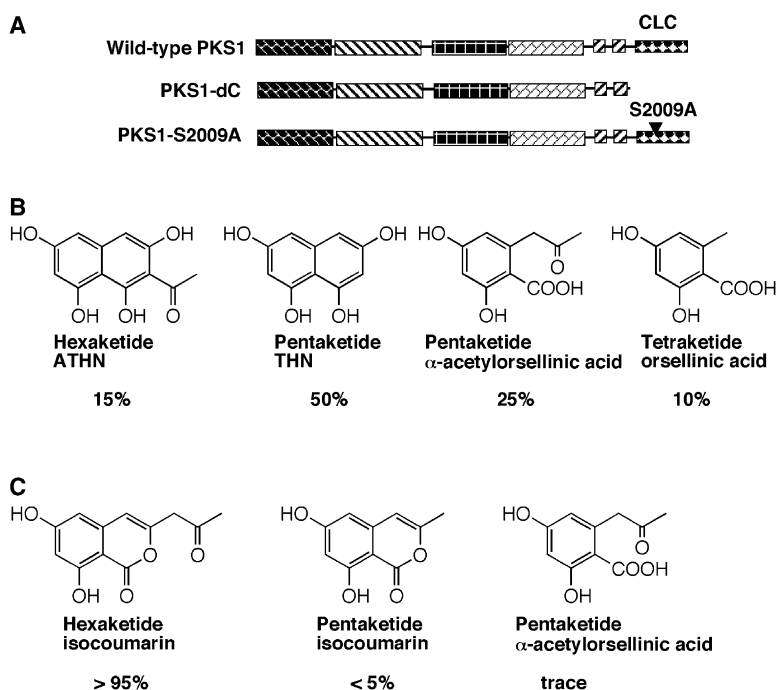
In this investigation to elucidate the function of the C-terminal TE-like domain of PKS1, PKS1 mutants on the C-terminal TE-like domain, PKS1-dC, and PKS1-S2009A were constructed (Figure 2A). PKS1-dC is a C-terminal TE-like domain-deletion mutant. PKS1-S2009A is a site-directed mutant of PKS1, in which the original

Ser2009 that corresponds to the active Ser site of WA CLC was mutated to Ala.

## Results

### Transformation

Expression plasmids pTA-*pkS1*-dC and pTA-*pkS1*-S2009A were constructed to express PKS1-dC and PKS1-A2009A,



**Figure 2. Product Identification of Wild-Type PKS1 and Its Mutants**

The proportion of each compound is shown as the percentage in the total of products. (A) PKS1 mutants. (B) Products of wild-type PKS1. (C) Products of PKS1-dC and PKS1-S2009A. ATHN, 2-acetyl-1,3,6,8-tetrahydronaphthalene; THN, tetrahydronaphthalene.

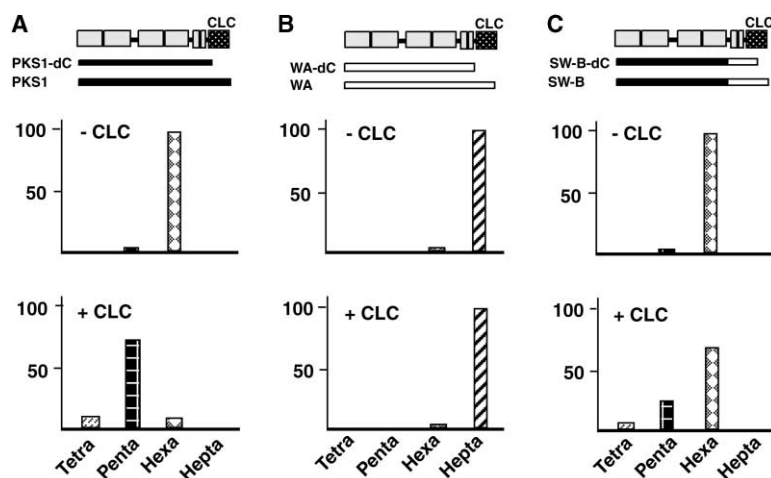


Figure 3. Effect of the CLC Domain on Chain Length

The black bar shows the part derived from PKS1 and the white bar shows that derived from WA. The length of bars indicates the percentage in the total of products. + CLC, wild-type in which the CLC domain is active; -CLC, mutant in which the CLC domain is inactive; Tetra, tetraketide; Penta, pentaketide; Hexa, hexaketide; Hepta, heptaketide. (A) PKS1; (B) WA; (C) SW-B.

respectively. These expression plasmids were introduced into *A. oryzae* M-2-3 to obtain transformants A.o/pTA-*pkS1*-dC and A.o/pTA-*pkS1*-S2009A.

The transformants with PKS1-dC or PKS1-S2009A formed white colonies, while transformants with wild-type PKS1 formed dark brown colonies.

#### Product Identification of Wild-Type PKS1

As a result of liquid chromatography-electrospray ionization-mass spectrometry (LC-ESIMS) analysis of the induction medium of the transformant with wild-type PKS1, unknown compounds were identified as a hexaketide 2-acetyl-1,3,6,8-tetrahydroxynaphthalene and its sulfate conjugate. The relative amounts of these PKS1 products, estimated by the peak area in the high-performance liquid chromatography (HPLC) profile monitored by UV absorption at 254 nm, are shown in Figure 2B. The HPLC profile shows that the pentaketide synthase PKS1 also produces notable amounts of tetraketide and hexaketide.

#### Product Identification of CLC Domain Mutants of PKS1

Both transformants with PKS1-dC or PKS1-S2009A yielded an almost identical product pattern and predominantly synthesized a hexaketide isocoumarin (ca. 95% of total products) with a pentaketide isocoumarin (ca. 5%) and only a trace amount of pentaketide  $\alpha$ -acetylorsellinic acid (Figure 2C). The lack of production of Claisen cyclization-type compounds and the predominance of pentaketide isocoumarin over pentaketide  $\alpha$ -acetylorsellinic acid in these mutants indicate that the CLC domain of PKS1 catalyzes the hydrolysis of ACP-bound thioesters as well as Claisen-type cyclization unlike the CLC domain of WA. The most striking result, however, was that the chain length of the products varied markedly depending on the presence or absence of the active C-terminal CLC domain.

#### Effect on Product Chain Length of the CLC Domain

In the case of WA, both the wild-type and CLC domain mutants specifically produce a heptaketide, which indicates that the WA CLC domain is not involved in product chain length (Figure 3B). On the other hand, in the case

of PKS1, the CLC domain mutants specifically produce a hexaketide, while the wild-type in which the CLC domain is active produces pentaketides as major products (Figure 3A). This indicates that PKS1 potentially has the capacity to synthesize hexaketides but that the active CLC domain interferes with chain length growth.

#### Product Identification of a CLC Domain Mutant of SW-B

Unlike PKS1-dC, SW-B produces significant amounts of pentaketides, indicating that the WA CLC domain in SW-B also shortens product chain length. To confirm this, we expressed SW-B-dC, which is a CLC domain-deletion mutant of SW-B. The transformant with SW-B-dC specifically produced hexaketide isocoumarin, like PKS1-dC and PKS1-S2009A (Figure 3C). This confirms that the WA CLC domain also shortens product chain length when fused with PKS1, while it does not in wild-type WA.

#### Discussion

The present results indicate that PKS1 potentially has the capacity to synthesize products as large as hexaketides but that the active CLC domain interferes with chain length growth. To the best of our knowledge, no domains or components that interfere with chain length growth have been reported previously among the known PKSs, including plant and bacterial PKSs.

In the biosynthesis of short-chain fatty acids in animals, the monofunctional thioesterase TE II has been reported to be responsible for the chain shortening of C16 acid to C10–C14 acids [17, 18]. By interacting with type I fatty acid synthase, which normally synthesizes C16 acid, TE II hydrolyzes shorter fatty acyl intermediates during condensation cycles. The CLC domain of PKS1, like TE II, is assumed to intercept the polyketide-methylene intermediate from the ACP half-way through the condensation reaction, and Ser2009 must play an important role in chain shortening.

As the CLC domain-deletion mutants of WA and PKS1 specifically synthesize heptaketide and hexaketide isocoumarins, respectively, it is obvious that the chain shortening by the CLC domain of PKS1 is not an essen-

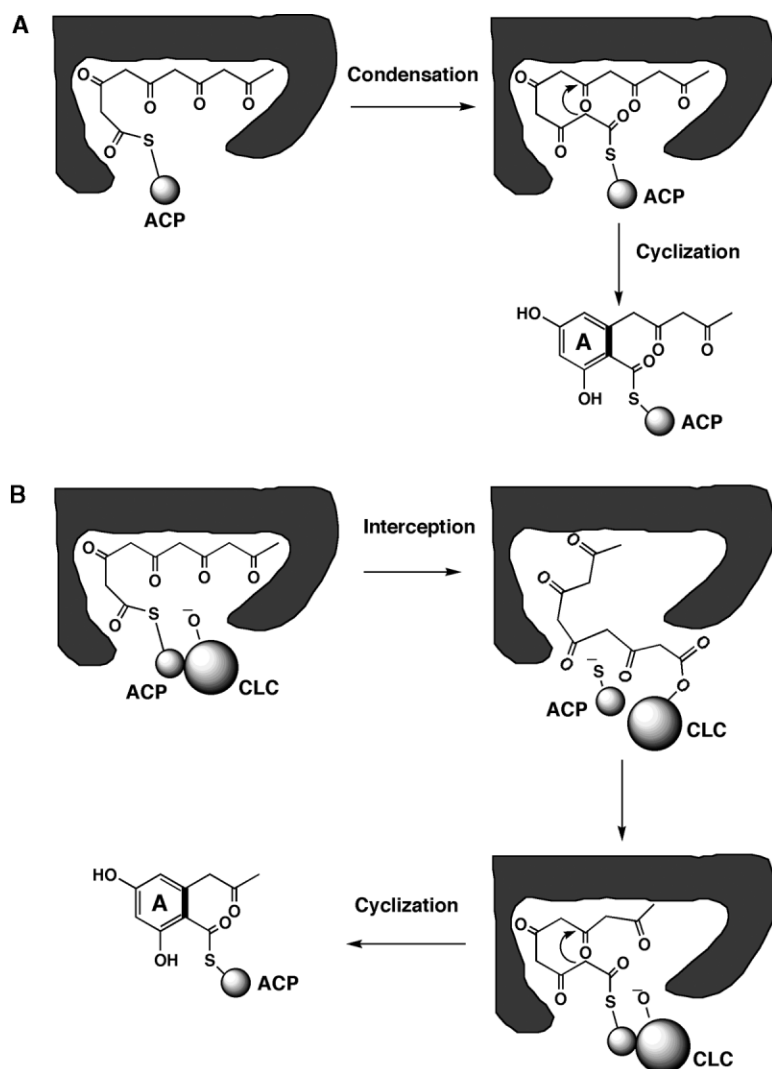


Figure 4. Proposed Mechanism of Chain Length and A-Ring Cyclization Controls

(A) CLC domain-inactivated mutants of PKS1. (B) Wild-type PKS1 where the CLC domain is active.

tial but an additional system controlling chain length, and that the fundamental chain length control and formation of the A-ring must be carried out by other domains such as the KS domain. Interestingly, however, A-ring cyclization of shorter chain intermediates, resulting from chain elongation intercept by the wild-type PKS1 and SW-B, occurs between C-2 and C-7, a position identical to that for hexaketide isocoumarin synthesis by the CLC domain-deletion mutant of PKS1. The polyketomethylene intermediate must be cyclized after being intercepted by the CLC domain, but A-ring formation is unlikely to be catalyzed by the CLC domain, as it is not an inherent function.

Based on the above evidence, we propose the following mechanism to account for the chain length shortening exhibited by the CLC domain. In the case of PKS1-dC (Figure 4A), the polyketomethylene intermediate grows to fill the active site cavity formed within the KS domain, and then A-ring cyclization occurs after the hexaketide intermediate is appropriately folded. In the case of wild-type PKS1 (Figure 4B), the shorter polyketomethylene (pentaketide) intermediate is transferred from the ACP domain to the CLC domain before the final condensation and pulled out of the cavity. This transfer

could be a reversible process, allowing the intermediate to be transferred back to the ACP domain and enter the cavity again. However, as the intermediate is a long and flexible chain, it is caught within the inner wall of the cavity and folded to form the A-ring. This results in the production of shorter chain products.

In this mechanism, the question is why the shorter intermediate is intercepted by the CLC domain in PKS1, but not intercepted in WA. A simple answer to this question could be that the PKS1 CLC domain has high specificity toward linear pentaketide intermediate, while the WA CLC domain shows low specificity toward shorter linear intermediates. However, as shown in Figure 3C, SW-B produces significant amount of pentaketide unlike SW-B-dC, which indicates that the WA CLC domain also interferes with chain length growth when fused with PKS1, while it does not in the wild-type WA. This fact suggests that the chain shortening function cannot be ascribed to the substrate specificity of the CLC domain alone, but is highly dependent on its interaction with other domains such as the KS and ACP. In the case of PKS1, the active site Ser residue of the CLC domain is assumed to be positioned near the ACP-bound thioester during the condensation reaction, while it is not in WA.

In the case of SW-B, the WA CLC domain is assumed to interact with ACP-bound thioester during the condensation reaction less efficiently than the PKS1 CLC domain in PKS1. It is probable that the substrate specificity of the CLC domain itself toward linear intermediate also influences the ratio of shorter chain products. Further experimental data including kinetic ones would clarify this interesting issue.

The results of this investigation also cast new light on the biosynthesis of THN in fungi. THN is a precursor of 1,8-dihydroxynaphthalene-melanin, a well-known virulence factor of plant [19]- and human [20]-pathogenic fungi. In the human-pathogenic fungus *Aspergillus fumigatus* that causes pulmonary aspergillosis, THN is synthesized by two discrete enzymes. ALB1 PKS, which shows high homology with WA, first synthesizes the heptaketide naphthopyrone from acetates, and then esterase-like AYG1 decomposes it into acetoacetate and pentaketide THN [21, 22]. On the other hand, in the phytopathogenic fungus *C. lagenarium* that causes anthracnose in cucurbitaceous plants, THN is directly synthesized by the single enzyme PKS1. In the present study, we have shown that PKS1 has the potential to synthesize hexaketide and its CLC domain intercepts the pentaketide intermediate from the ACP before the final condensation reaction, resulting in the synthesis of pentaketide THN. It is an intriguing question to ask why these fungi use such complicated mechanisms to synthesize THN instead of producing the pentaketide directly and specifically.

## Significance

The understanding of how PKSs control their PKS reactions such as chain length determination is expected to contribute to the production through bioengineering of novel polyketides possessing clinically important activities. In this study, we found that the PKS1 CLC domain has multiple functions such as Claisen-type cyclization, hydrolysis, and chain shortening, unlike the WA CLC domain. The chain shortening function of the CLC domain is unprecedented among other types of PKSs, including plant and bacterial PKSs. The functional diversity of the CLC domain directly affects the structural diversity of product polyketides in *C. lagenarium* PKS1. The CLC domain would thus be an important target for bioengineering. Although PKS1 has domain architecture identical to that of WA, PKS1 possesses a unique mechanism for chain length determination as described above, demonstrating the mechanistic diversity of fungal aromatic PKSs. Furthermore, we found that the WA CLC domain also shortens product chain length when fused with PKS1. These results indicate that domain-domain interactions influence the mechanistic diversity of fungal aromatic PKSs. It is important to understand such interactions as well as to understand the function of each domain in fungal aromatic PKSs.

## Experimental Procedures

### Transformation

Each PKS gene was cloned into the fungal expression vector pTAex3. Each expression plasmid was introduced into the heterolo-

gous fungus *A. oryzae* M-2-3, which is an arginine auxotrophic mutant produced using the protoplast-polyethylene glycol method [23, 24].

### Expression of PKSs

The transformants were selected on minimal agar plates. After pre-culture for 4 days, the transformants were shake cultured, first in Czapek-Dox medium supplemented with glucose for 4 days and then in Czapek-Dox medium supplemented with starch for induction for 1 additional day. In the case of transformants with SW-B or SW-B-dC, expression was induced by shaking the culture in minimal medium supplemented with starch for induction for 1 additional day, after shaking the culture in Czapek-Dox medium supplemented with glucose for 4 days. Product characterization of these mutants was performed by LC-ESIMS analysis of the culture medium after induction.

### Construction of CLC Domain Mutant Genes

The pTA-pks1-dC was created by digesting the expression plasmid pTAPSG [15] with restriction enzyme SmaI (TaKaRa), followed by self-ligation.

The pTA-pks1-S2009A was generated by site-directed mutagenesis using the PCR method. The PCR primers used were 5'-CCGCCC GCAGCCCAACCC-3', 5'-CCACCAAGGAGATCAACACTC-3', and 5'-TACGCGTCTAGAAGGAACGTGTAGATTG-3'. The PCR fragment was subcloned into pT7 Blue vector to construct pT7-S2009A. The SmaI fragment of pT7-S2009A was replaced with the SmaI fragment of pTAPSG to construct pTA-pks1-S2009A.

The pTA-sw-B-dC was created by digesting the pTA-sw-B with restriction enzyme MluI (TaKaRa), followed by end blunting and self-ligation.

### Characterization of PKS Products

Characterization of each PKS was performed using HPLC and LC-ESIMS analysis of the culture medium after induction. A reverse-phase column (TOSOH ODS-80Ts, 4.6 × 150 mm) was eluted with a linear gradient of 5%–40% CH<sub>3</sub>CN in 2% AcOH (aqueous solution) over 30 min at a flow rate of 0.8 ml/min, with detection at 254 nm. The same LC conditions were employed for LC-ESIMS (LCQ, Thermo Quest) with negative ion monitoring. Chemical identification was done by direct comparison with authentic samples.

## Acknowledgments

We are grateful to Drs. M.E. Mayorga (Millennium Pharmaceuticals Inc.) and W.E. Timberlake (Cereon Genomics) for providing the *A. nidulans* wA gene, to Professor Y. Kubo (Kyoto Prefectural University) for providing the *C. lagenarium* pks1 gene, and to Professor K. Gomi (Tohoku University, Sendai, Japan) for his kind help in fungal expression. This work was financially supported by a Grant-in-Aid for Scientific Research on Priority Area (A) (No. 12045213) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Grant-in-Aid for Scientific Research (S) (No. 15101007) from the Japan Society for the Promotion of Science (JSPS). A.W. is a recipient of a JSPS young researcher fellowship.

Received: February 8, 2004

Revised: April 28, 2004

Accepted: May 19, 2004

Published: August 20, 2004

## References

1. Kao, C.M., Luo, G., Katz, L., Cane, D.E., and Khosla, C. (1995). Manipulation of macrolide ring size by directed mutagenesis of a modular polyketide synthase. *J. Am. Chem. Soc.* 117, 9105–9106.
2. Fujii, I., Watanabe, A., Tada, H., Ono, Y., Mori, Y., Kajimoto, S., Yasuoka, Y., and Sankawa, U. (2002). Architecture and functional analysis of fungal polyketide synthases. *Mycotoxins* 52, 135–142.
3. Hutchinson, C.R., and Fujii, I. (1995). Polyketide synthase gene manipulation: a structure-function approach in engineering novel antibiotics. *Annu. Rev. Microbiol.* 49, 201–238.

4. Austin, M.B., and Noel, J.P. (2003). The chalcone synthase superfamily of type III polyketide synthase. *Nat. Prod. Rep.* 20, 79–110.
5. Bisang, C., Long, P.F., Cortes, J., Westcott, J., Crosby, J., Matharu, A.L., Cox, R.J., Simpson, T.J., Staunton, J., and Leadlay, P.F. (1999). A chain initiation factor common to both modular and aromatic polyketide synthases. *Nature* 401, 502–505.
6. Ferrer, J.L., Jez, J.M., Bowman, M.E., Dixon, R.A., and Noel, J.P. (1999). Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. *Nat. Struct. Biol.* 6, 775–784.
7. Jez, J.M., Austin, M.B., Ferrer, J.L., Bowman, M.E., Schroder, J., and Noel, J.P. (2000). Structural control of polyketide formation in plant-specific polyketide synthases. *Chem. Biol.* 7, 919–930.
8. Beck, J., Ripka, S., Signer, A., Schiltz, E., and Schweizer, E. (1990). The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. *Eur. J. Biochem.* 192, 487–498.
9. Fujii, I., Ono, Y., Tada, H., Gomi, K., and Ebizuka, Y. (1996). Cloning of the polyketide synthase gene *atX* from *Aspergillus terreus* and its identification as the 6-methylsalicylic acid synthase gene by heterologous expression. *Mol. Gen. Genet.* 253, 1–10.
10. Mayorga, M.E., and Timberlake, W.E. (1992). The developmentally regulated *Aspergillus nidulans* *wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. *Mol. Gen. Genet.* 235, 205–212.
11. Takano, Y., Kubo, Y., Shimizu, K., Mise, K., Okuno, T., and Furusawa, I. (1995). Structural analysis of PKS1, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium*. *Mol. Gen. Genet.* 249, 162–167.
12. Watanabe, A., Fujii, I., Sankawa, U., Mayorga, M.E., Timberlake, W.E., and Ebizuka, Y. (1999). Re-identification of *Aspergillus nidulans* *wA* gene to code for a polyketide synthase of naphthopyrone. *Tetrahedron Lett.* 40, 91–94.
13. Fujii, I., Watanabe, A., Sankawa, U., and Ebizuka, Y. (2001). Identification of Claisen cyclase domain in fungal polyketide synthase *WA*, a naphthopyrone synthase of *Aspergillus nidulans*. *Chem. Biol.* 8, 189–197.
14. Watanabe, A., Ono, Y., Fujii, I., Sankawa, U., Mayorga, M.E., Timberlake, W.E., and Ebizuka, Y. (1998). Product identification of polyketide synthase coded by *Aspergillus nidulans* *wA* gene. *Tetrahedron Lett.* 39, 7733–7736.
15. Fujii, I., Mori, Y., Watanabe, A., Kubo, Y., Tsuji, G., and Ebizuka, Y. (1999). Heterologous expression and product identification of *Colletotrichum lagenarium* polyketide synthase encoded by the *pks1* gene involved in melanin biosynthesis. *Biosci. Biotechnol. Biochem.* 63, 1445–1452.
16. Watanabe, A., and Ebizuka, Y. (2002). A novel hexaketide naphthalene synthesized by a chimeric polyketide synthase composed of fungal pentaketide and heptaketide synthases. *Tetrahedron Lett.* 43, 843–846.
17. Renobales, M.D., Rogers, L., and Kolattukudy, P.E. (1980). Involvement of a thioesterase in the production of short-chain fatty acids in the uropygial glands of mallard ducks (*Anas platyrhynchos*). *Arch. Biochem. Biophys.* 205, 464–477.
18. Witkowska, H.E., Green, B.N., and Smith, S. (1990). The carboxyl-terminal region of thioesterase II participates in the interaction with fatty acid synthase. *J. Biol. Chem.* 265, 5662–5665.
19. Yamaguchi, I., and Kubo, Y. (1992). Target sites of melanin biosynthesis inhibitors. In *Target Sites of Fungicide Action*, W. Koller, ed. (London: CRC Press), pp 101–118.
20. Kwon-Chung, K.J., and Bennett, J.E. (1992). *Aspergillosis*. In *Medical Mycology* (Baltimore: Williams and Wilkins), pp 201–247.
21. Watanabe, A., Fujii, I., Tsai, H.-F., Chang, Y.C., Kwon-Chung, K.-J., and Ebizuka, Y. (2000). *Aspergillus fumigatus* *alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. *FEMS Microbiol. Lett.* 192, 39–44.
22. Tsai, H.-F., Fujii, I., Watanabe, A., Wheeler, M.H., Chang, Y.C., Yasuoka, Y., Ebizuka, Y., and Kwon-Chung, K.-J. (2001). Pentaketide-melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. *J. Biol. Chem.* 276, 29292–29298.
23. Gomi, K., Iimura, Y., and Hara, S. (1987). Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans* *argB* gene. *Biosci. Biotechnol. Biochem.* 59, 1869–1874.
24. Fujii, T., Yamaoka, H., Gomi, K., Kitamoto, K., and Kumagai, C. (1995). Cloning and nucleotide sequence of the ribonuclease T1 gene (*rntA*) from *Aspergillus oryzae* and its expression in *Saccharomyces cerevisiae* and *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* 59, 1869–1874.